Identifying the Highest Affinity Nanobody Binder for RHC-1 to Investigate Its Effect on Plants' Stomatal Mechanism

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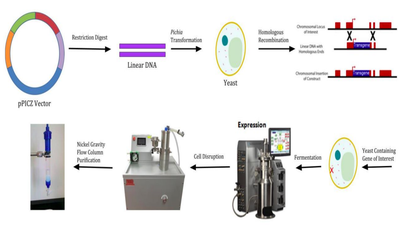
Introduction

RHC-1 is a member of the multidrug and toxic compound extrusion (MATE) family of transporters. Its role is to repress HT1, which is a protein that inhibits CO2-induced stomatal closing. Stomatal opening and closing is an important part of a plant's metabolism because stomata open in response to low CO2 concentrations and close in high CO2 concentrations. With CO2 levels constantly rising, stomatal movement is being affected. However, not much is known about how CO2 sensing by the plant affects stomatal activity. Studying RHC-1 is essential because of its large role in stomatal activity and since CO2 levels are rising, there will be changes to stomatal activity which could lead to unknown consequences. By propagating yeast cells containing the protein, screening for the best expressors, and purifying the protein, we can find a nanobody binder to RHC-1 that has the highest affinity, which will increases the chances of modulating the activity of RHC-1 the most.

Pichia Pastoris Transformation and Protein Purification

In order to get our gene of interest to express in a yeast cell, transformation of our DNA construct (plasmid) into *E. coli* was first necessary. From there, the *E. coli* was grown in culture to magnify the amount of our plasmid harboring our desired gene, which was then isolated from the bacterial cells using a plasmid mini-prep kit. Through electroporation, the DNA was taken up by the yeast cells, after linearization of our plasmid, using the restriction enzyme *Pme*1. By carefully controlling factors such as dissolved oxygen, agitation and pH, the yeast was fermented in a BioFlo 415 Bioreactor and protein expression was induced with methanol. These cells were subsequently harvested and disrupted with a French Press. The cells were spun in a centrifuge, the supernatant was discarded and the pellet was kept ensuring only the membrane fraction was left. To isolate the protein, affinity chromatography was carried out by utilizing a His-60 gravity nickel column. In order to further purify the protein, size exclusion chromatography was carried out using FPLC.

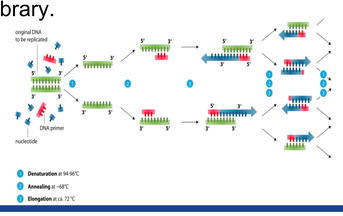


Cell Sorting

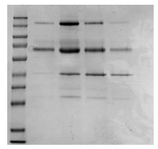
Bacteria cells were sorted in the BioRad S3e Cell Sorter based on their fluorescence. Cells that reached a certain threshold of fluorescence were guided into a vial while all others were discarded. Cells were selected for their ability to display a binder on its surface that bound our protein of interest (the antigen) that was fused with green fluorescent protein (GFP). We then re-sorted the cells to select for the tightest binders, as some interactions are transient and unstable. In this way, we were able to collect those binders that had the most desirable affinities.

Recombination

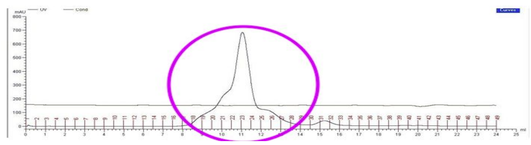
Through recombination, stronger and stronger binders can be produced. To recombine, DNA polymerases, template DNA and primers were mixed and put in a PCR (polymerase chain reaction) machine which multiplied the amount of DNA available to work with. A gel was ran and the concentration of DNA was measured. The DNA was ligated into pBAD vector, then, via electroporation, the DNA was taken up by shuffle T7 competent cells and plated overnight to produce a new generation of library.



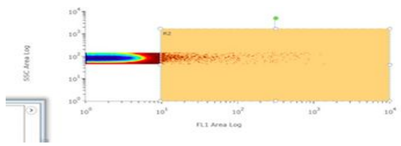
Result



The leftmost lane is the marker that serves as a reference for molecular weight. The three following lanes are washes increasing imidazole concentrations and the farthest right lane is the elution. This gel was ran after doing affinity chromatography

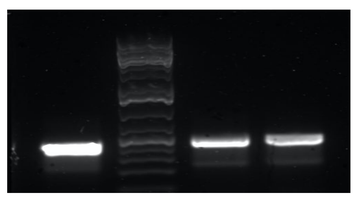


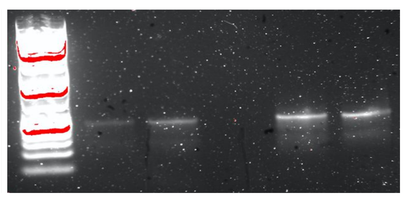
The purple circle indicates where the protein separated from the contaminants during size exclusion chromatography

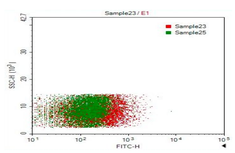


The cells contained within the yellow rectangle are those that met the minimum fluorescence to be sorted into the vial of cells that would be further studied

The image to the bottom left is showing that the insert is present and ready to be recombined. The image to the bottom right shows that the recombination was done successfully. There are two different regions because two reactions were carried out.

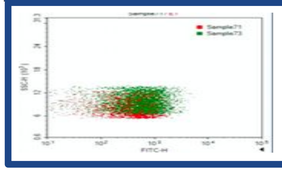






The image to the left shows red dots which showed a substantial shift to the right which shows that this sample was a good expressor of our target.

Results (cont.)

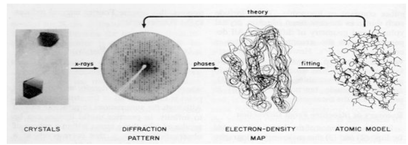


Conversely, the image to the left shows a yeast sample that did not shift substantially and was not a good expressor of our target

Conclusion

RHC-1's crucial role in stomatal opening makes it an important transporter in plants as stomata regulate the amount of CO2 absorbed. This is of importance, as CO2 is essential for photosynthesis. When CO2 levels are high, a protein called HT1 negatively regulates the ability of the stomata to close, but this is detrimental to the plant. Keeping stomata open while there is sufficient CO2 in the plant allows water to escape. But, RHC-1 represses HT1 so that the stomata can close when there is enough CO2, allowing the plant to effectively budget its resources.

Through our research, we now have generated a number of nanobody binders of RHC-1. The next logical step would be to purify the nanobody and test its affinity for RHC1. In purifying it, crystallization trials can also be carried out with and without RHC-1 bound. Crystallizing it would allow us to use X-ray diffraction to identify the structure of our nanobody. Next, we could pinpoint the sites at which RHC-1 binds to the nanobody, and use this information to introduce mutations that may increase binding to RHC-1 and, possibly, modulate the transporters function. The end goal would be to introduce our nanobody to actual plants and see if the effects observed in the lab translate to an in vivo study.



Reference

1. Tian, Wang, et al. "A Molecular Pathway for CO2 Response in Arabidopsis Guard Cells." *Nature Communications*, vol. 6, no. 1, 20 Jan. 2015, doi:10.1038/ncomms7057.
2. Doshi, Rupak, et al. "Functional Characterization and Discovery of Modulators of SbMATE, the Agronomically Important Aluminium Tolerance Transporter from Sorghum Bicolor." Scientific Reports, vol. 7, no. 1, 2017, doi:10.1038/s41598-017-18146-8.

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